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REPLY TO ATTENTION OF:
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AD B177316

29 Aug 95

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Building 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limited distribution statement on technical report for Contract Number DAMD17-90-C-0082. Request the limited distribution statement for AD Number ADB177316 be changed to "Approved for public release; distribution unlimited." A copy of this report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus, DSN 343-7322.

for Phyllis Rinehart
GARY R. GILBERT
COL, MS
Deputy Chief of Staff
for Information Management

AD_____

CONTRACT NO: DAMD17-90-C-0082

TITLE: TRI-ELISA FOR SIMULTANEOUS ANALYSIS OF THREE ANALYTES

PRINCIPAL INVESTIGATOR: Sheryl Gregg Hohle, M.S.,M.S.-M.O.T.

CONTRACTING ORGANIZATION: Bio-Metric Systems, Inc.
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13. ABSTRACT *(Maximum 200 words)*

The goal of this research effort is to develop a three-enzyme ELISA system in which three malarial analytes can be quantitated concurrently from a single biological sample. During the Phase I effort, we were able to develop a non-quantitative tri-ELISA that was capable of differentiating between three *Plasmodium* antigens/antibodies previously identified and optimized by Dr. Robert Wirtz at the Walter Reed Army Institute of Research. During this Phase II effort, we expect to improve and optimize the system, as well as provide both antibody pre-coated microplates and final kits for analysis by Dr. Wirtz. The resulting system will provide an efficient and potentially productive approach for improving an existing diagnostic assay for malarial sporozoites. The assay can be used by the World Health Organization for epidemiological studies designed to monitor, and through preventative mechanisms, potentially eradicate a disease which currently threatens greater than 56% of the world's population.

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PREFACE

This final report is submitted to the U.S. Army Medical Research Acquisition Activity as a summary of work conducted for the Phase II contract No. DAMD17-90-C-0082, entitled "TRI-ELISA FOR SIMULTANEOUS ANALYSIS OF THREE ANALYTES" issued from Walter Reed Army Institute of Research (Dr. Robert Wirtz, Contracting Officer's Technical Representative). It contains a logical, efficient and potentially productive approach for improving an existing analytical assay for malarial sporozoites in mosquitos. The resulting assay can be used by the World Health Organization for epidemiological studies designed to monitor, and through preventative mechanisms, potentially eradicate this disease which currently threatens approximately 56% of the world's population.

As Contractor, Bio-Metric Systems, Inc, independently and not as an agent of the Government, has furnished the necessary personnel, facilities, equipment and supplies, and otherwise exerted its best efforts to do all the things necessary for or incident to the accomplishment of the Government's requirement set forth in the subject Contract.

Bio-Metric Systems, Inc., (BSI) offers personnel, experience and facilities highly suitable for the assessment and advancement of the "state of the art" in surface modification, as it pertains to biological, toxin and chemical agent detection or to improvements in biocompatibility of implantable medical devices. Questions concerning the technical aspects of this work effort should be directed to the undersigned or to Sheryl G. Hohle, Principal Investigator. For administrative matters, please contact the undersigned.

Patrick E. Guire

Patrick E. Guire,
Senior Vice President/Chief Scientific Officer

ABBREVIATIONS USED IN THIS REPORT

Antigens:

P_v210	*Plasmodium vivax* variant 210
P_f	*Plasmodium falciparum*
P_v247	*Plasmodium vivax* variant 247
BC	boiled casein
CS	circumsporozoite

Enzymes and Substrates:

β-gal	β-galactosidase
ONPG	o-nitrophenyl galactopyranoside (β-gal substrate)
AP	alkaline phosphatase
HRP	horseradish peroxidase
TMB	3,3',5,5'-tetramethylbenzidine (HRP substrate)

Buffer:

TNT	50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.2

I. Purpose and Scope of Research Effort.

The goal of this research effort was to develop a three-enzyme ELISA system in which three malarial analytes can be quantitated concurrently from a single biological sample. During the Phase I effort, we were able to develop a non-quantitative tri-ELISA that was capable of differentiating between three *Plasmodium* antigens/antibodies previously identified and optimized by Dr. Robert Wirtz at the Walter Reed Army Institute of Research. During this Phase II effort, we improved and optimized the system, and provided both antibody pre-coated microplates and final kits for analysis by Dr. Wirtz. The resulting system provides an efficient and potentially productive approach for improving an existing diagnostic assay for malarial sporozoites. The assay can be used by the World Health Organization for epidemiological studies designed to monitor, and through preventative mechanisms, potentially eradicate a disease which currently threatens greater than 56% of the world's population.

II. Overall Progress.

The technical objectives of this Phase II project were to: 1) optimize chemistries enabling the immobilization of monoclonal capture antibodies; 2) determine stability of the capture antibodies on the pre-coated plate; 3) reduce non-specific protein adsorption, and therefore improve signal to noise ratio and reduce the number of steps in the ELISA procedure; 4) optimize assay conditions for the simultaneous measurement of three enzyme/chromogen systems; 5) determine stability of the antibody, antigen and substrate components for the final diagnostic kit system; 6) determine detection limits of the tri-ELISA using mosquitos and human serum; and 7) prepare test kits for independent laboratory evaluation of mosquitos infected with *Plasmodium* (detecting sporozoites) and human serum samples (detecting anti-*Plasmodium* antibodies). While the majority of this work was successfully completed, the DOD was unable to supply us with sporozoite samples or with human serum samples due to the associated health hazards. Therefore, objective number six was deferred to Dr. Wirtz at the Walter Reed Army Institute of Research.

This Phase II study was designed to answer the following questions: 1) Will pre-coated plates provide more stability, improved signal:noise ratio, and greater ease-of-use? 2) Can the three enzyme-antibody conjugates which we propose work at a sufficient activity level to be sensitive in the proposed format? 3) Are the optimal stabilities for each component and for the whole kit practical for the intended field use?; and, 4) Will the complete tri-ELISA assay kits demonstrate utility when tested with mosquitos and human serum samples? This final report outlines the affirmative answers to these issues and describes the preparation and use of the final malaria kit.

The malaria kit, shown in Figure 1 and detailed in the attached instruction "booklet," is actually comprised of three separate assays. The goal of this project was to prepare a kit which could be used for: 1) the visual or instrumented screening of mosquito samples for the presence of any one or all of three *Plasmodium* species; 2) the specific quantitative instrumented analysis of any "positive" sample for one of the three malarial sporozoites; and 3) the analysis of human serum samples which contain anti-*Plasmodium* IgG. We refer to these assays as the "Tri-ELISA," the "Quantitative ELISA," and the "Human Serum ELISA," respectively. Collectively, they are referred to as the "Malaria Kit." Each of the separate assays shares components with the other assays, thus making the kit format logical and efficient.

In designing the kit, several product attributes were considered desirable. First, we intended to make the system user-friendly, requiring as few steps as possible, with color-coded labels to prevent confusion. A second desirable characteristic was that the reagents should be relatively safe, with no highly toxic or carcinogenic compounds. Third, the completed assay should yield colors and color

combinations that can easily be visualized by eye, or that can be read conclusively using a plate reader; *i.e.*, no precipitating substrates, and the colors must be as close to primary colors as possible. Finally, the components must be stable to storage for at least 1 year at 4°C. Each of these attributes was successfully achieved during this Phase II project effort.

The development of a Malaria Kit required the optimization of 6 basic components: 1) antigens; 2) pre-coated antibody plates; 3) HRP-antibody conjugates; 4) enzyme conjugate cocktail; 5) HRP substrate; and 6) common substrate. Extensive studies were conducted on each component to determine optimal conjugation methods, stabilization chemistries, formats, and ease-of-use. The details of the many attempts are contained in previous technical progress reports. Final methods were chosen based on those chemistries that yielded optimal results when used in combination with other kit components. The reviewer can refer to the kit instruction booklet for details pertaining to each assay system, protocols, and component composition.

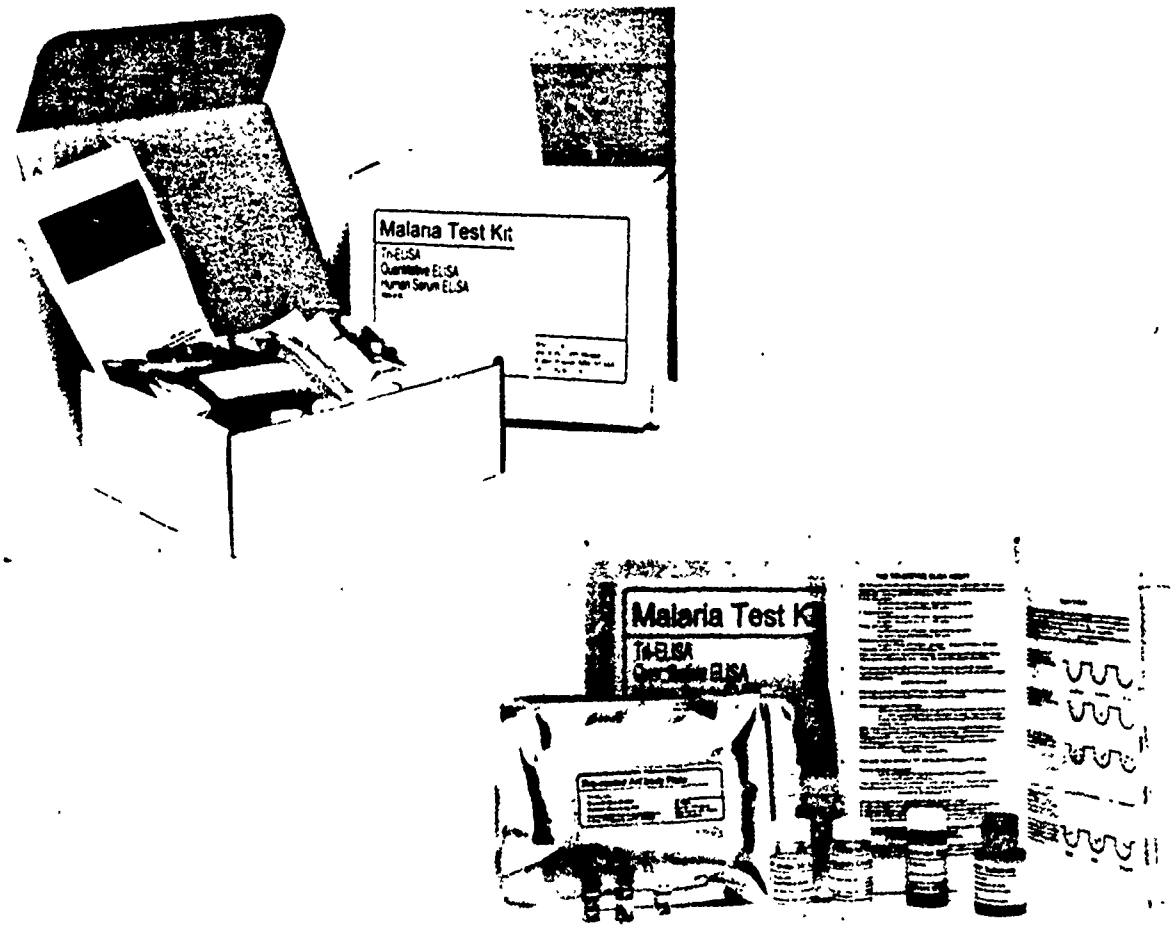


FIGURE 1. THE MALARIA TEST KIT. The complete kit, shown in the top photograph, is comprised of the following components: 2 pre-coated antibody plates, 8 plate sealers, 1 lyophilized *Pv*210 antigen (red), 1 lyophilized *Pf* antigen (yellow), 1 lyophilized *Pv*247 antigen (blue), 1 lyophilized enzyme conjugate cocktail, 1 liquid *Pv*210-HRP conjugate, 1 liquid *Pf* HRP conjugate, 1 liquid *Pv*247 HRP conjugate, 1 liquid anti human IgG HRP conjugate, 1 lyophilized common substrate, 1 HRP substrate, and detailed kit instructions. These components which are used for the tri-ELISA are shown in the lower photograph. All components contained in this kit are expected to remain stable for a minimum of 1 year when stored at 4°C

Final Malaria Kit Evaluation. Samples of the completed kits were evaluated for their sensitivity in both the Tri-ELISA and Quantitative ELISA formats. The Human Serum ELISA was not tested due to lack of sero-positive samples. However, the components of this assay were tested for background noise, and for activity using sero-negative human serum.

Tri-ELISA. The tri-ELISA was performed with both the kit antigens and freshly prepared antigens. Photographs of the completed tri-ELISA are shown in Figure 2. Graphed results of this same assay are shown in Figure 3. For this assay, substrate incubations were conducted for 30 minutes at 37°C for the common substrate and at RT for the HRP substrate. Using fresh antigens, the assay sensitivities in this screening test were 0.39 ng/ml for P_f and P_v210, and 7.8 ng/ml for P_v247 using the plate reader. By eye, color was observed down to the 0.78 - 1.56 ng/ml range for P_f and P_v210 and 15.6 ng/ml for P_v247.

Quantitative ELISA. An ELISA was also run according to the kit instructions, using both the kit antigens, and freshly prepared antigens. Results of the quantitative ELISA after incubation with TMB substrate at RT for 30 minutes are given in Figure 4. Using fresh antigens, the assay sensitivities were 3.13 pg/ml P_v210, 12.5 pg/ml P_f, and < 7.8 ng/ml P_v247. When analyzing these results, it is important to point out that the kit will most likely be used to test for the presence of malarial sporozoites; therefore the activity of BC-antigen does not necessarily reflect the ability of the assay to detect the natural analyte. One can see from the results that the lyophilized BC-P_v247 has lost activity relative to a non-lyophilized conjugate; the use of fresh BC-antigens is more representative of the assay's true sensitivity. An additional point should be made concerning this assay; the background absorbances are higher than typically seen. This is likely due to a faulty plate washer on the day this assay was run. If background color does become a problem in these assays, a blocking protein such as BSA may be added at 1% to improve the signal to noise ratio.

III. Problem Areas.

There are no problem areas.

IV. Work to be Accomplished.

All work is complete for this contract.

V. Administrative Comments.

There are no additional comments.

VI. Gantt Chart.

See attached.

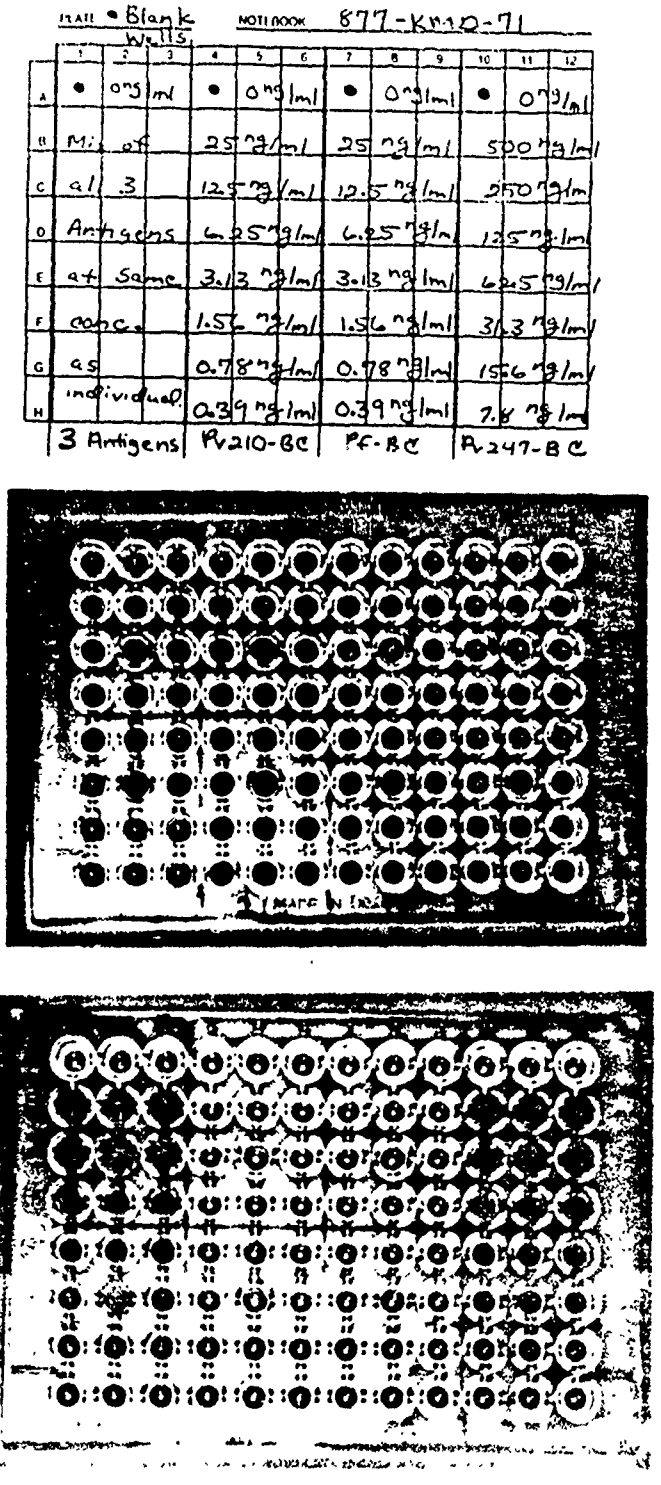


FIGURE 2. PHOTOGRAPHS OF COMPLETED TRI-ELISA. An assay using fresh antigen was run as diagrammed in the map shown at the top of the page. The common substrate was added to each well of the plate and allowed to incubate at 37°C for 30 minutes. After reading the plate at A570 and A405, and taking the first photograph (red, yellow), HRP substrate was added to the plate, and was developed for an additional 30 minutes, this time at RT. The plate was then read at A655, and the second (blue) photograph was taken. The data in Figure 3 corresponds to these plates.

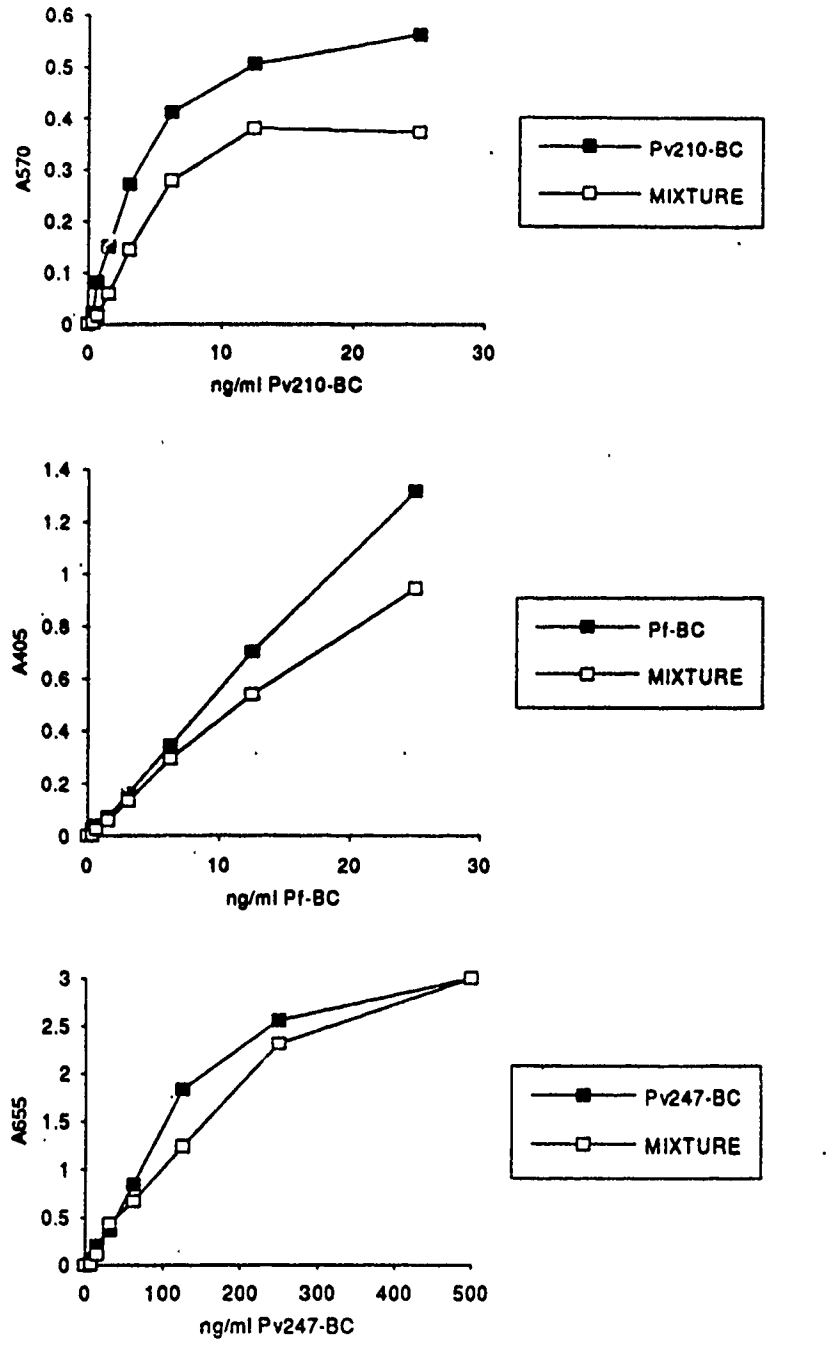


FIGURE 3. Tri-ELISA ANALYSIS -- FINAL KIT. Assays were conducted using the tri-enzyme ELISA malaria kit for the analysis of the three Plasmodium antigens. In each test, fresh non-lyophilized antigens were used both individually and as a mixture (Pf-BC, Pv210-BC, and Pv247-BC). The background (no antigen) absorbance values were as follows. for the Pv210-BC read at A570, 0.000, for the mixture read at A570, 0.000, for the Pf-BC read at A405, 0.000, for the mixture read at A405, 0.006 ± 0.000, for the Pv247-BC read at A655, 0.052 ± 0.006, and for the mixture read at A655, 0.081 ± 0.016. n=4 for all samples.

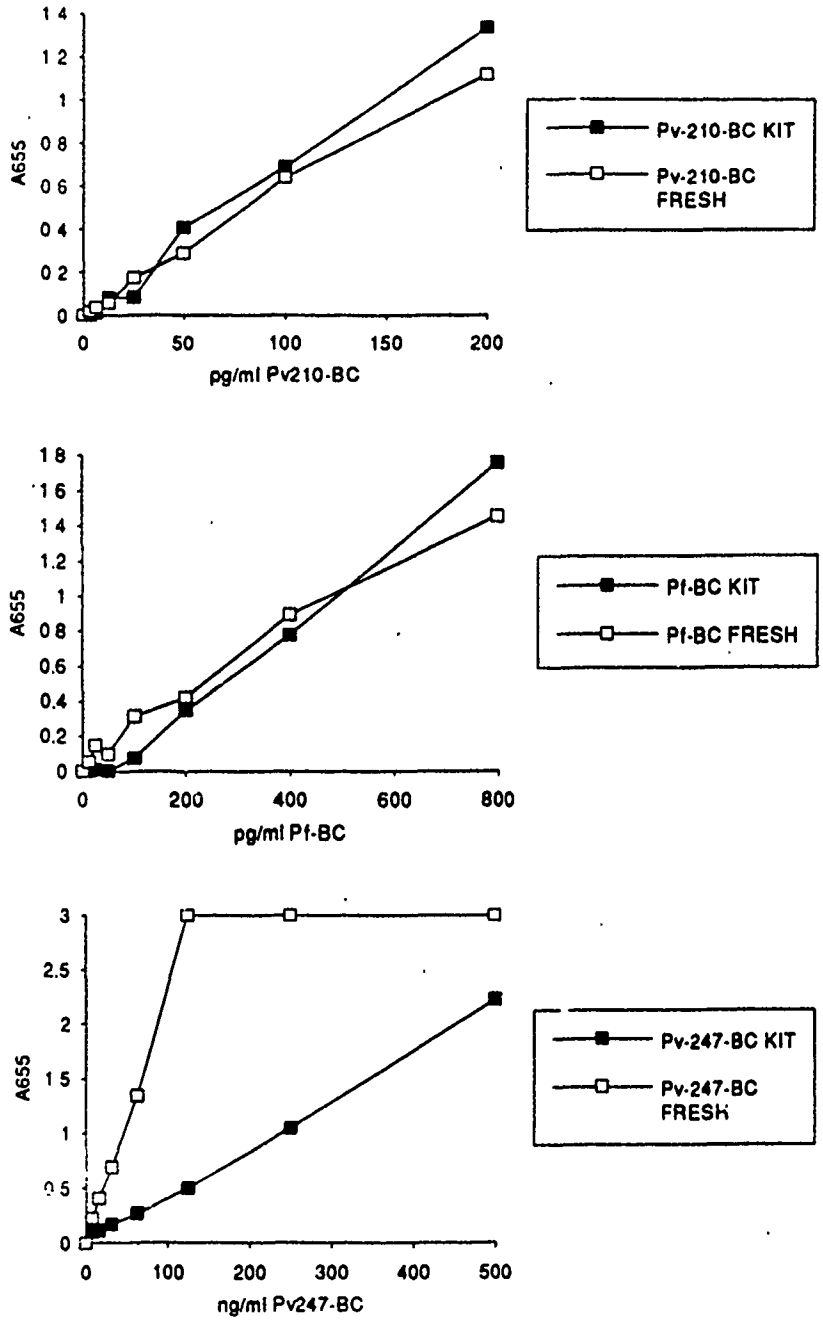
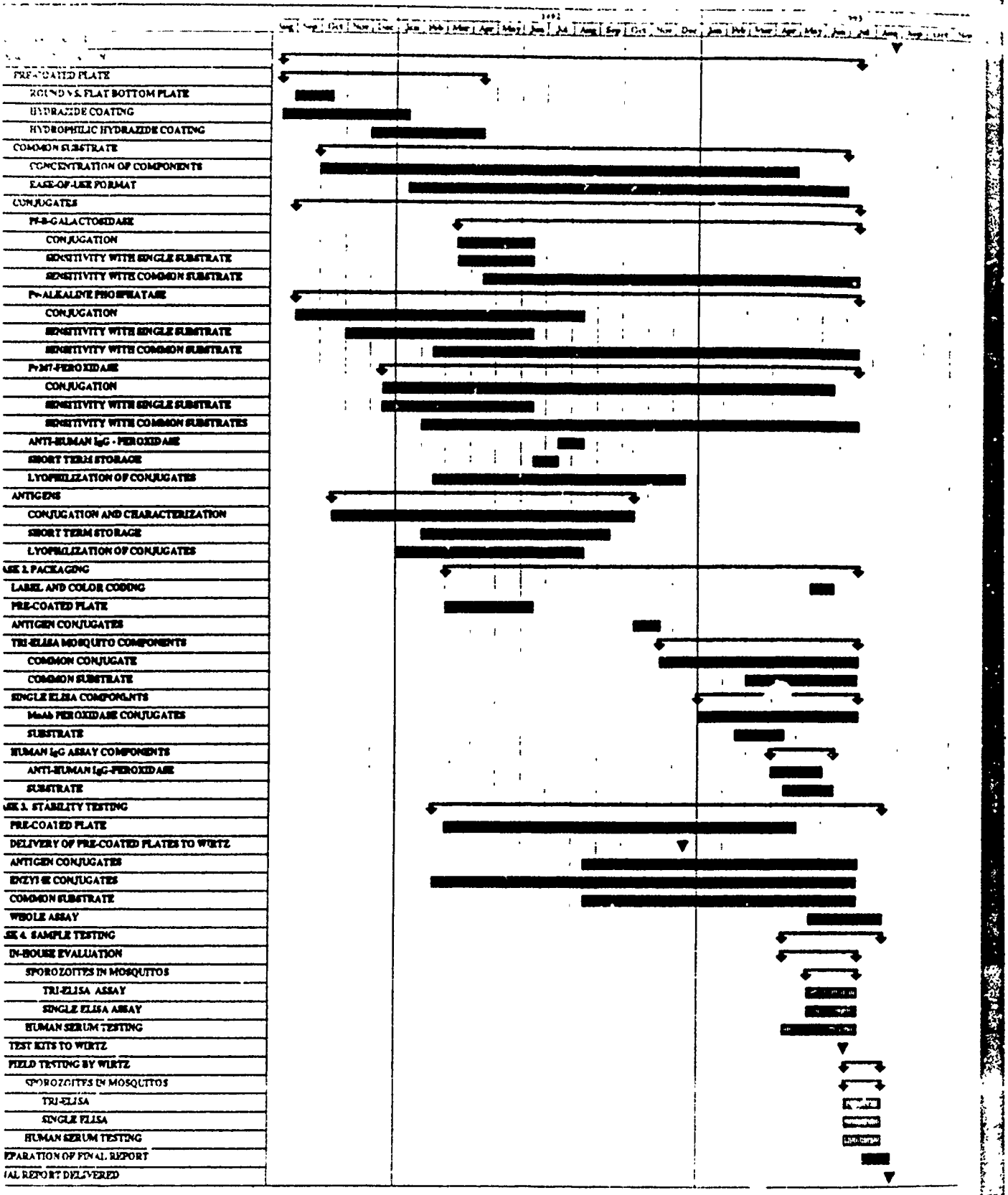
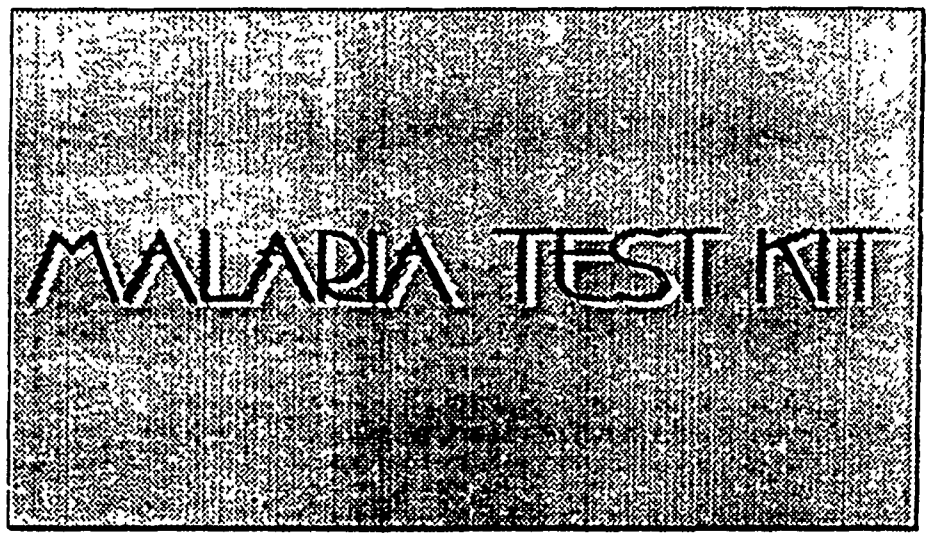


FIGURE 4. QUANTITATIVE ELISA -- FINAL KIT. Assays were conducted using the malaria kit for the quantitative analysis of the three Plasmodium antigens. The "KIT" lyophilized and reconstituted antigens were compared to the "FRESH" non-lyophilized antigens. The background (no antigen) absorbance values were as follows: for the Pv210 Kit, 0.163 ± 0.026; for the Pv210 fresh, 0.124 ± 0.011, for the Pf Kit, 0.418 ± 0.029, for the Pf fresh, 0.312 ± 0.009, for the Pv247 Kit, 0.161 ± 0.007, and for the Pv247 fresh, 0.191 ± 0.021. These unusually high backgrounds are most likely due to a faulty plate washer. n=4 for all samples.


PRE-COATED PLATE
ROUND VS. FLAT BOTTOM PLATE
HYDRAZIDE COATING
HYDROPHILIC HYDRAZIDE COATING
COMMON SUBSTRATE
CONCENTRATION OF COMPONENTS
EASE-OF-USE FORMAT
CONJUGATES
Pf-B-GALACTOSIDASE
CONJUGATION
SENSITIVITY WITH SINGLE SUBSTRATE
SENSITIVITY WITH COMMON SUBSTRATE
Pv-ALKALINE PHOSPHATASE
CONJUGATION
SENSITIVITY WITH SINGLE SUBSTRATE
SENSITIVITY WITH COMMON SUBSTRATE
Pv247-PEROXIDASE
CONJUGATION
SENSITIVITY WITH SINGLE SUBSTRATE
SENSITIVITY WITH COMMON SUBSTRATES
ANTI-HUMAN IgG - PEROXIDASE
SHORT TERM STORAGE
LYOPHILIZATION OF CONJUGATES
ANTIGENS
CONJUGATION AND CHARACTERIZATION
SHORT TERM STORAGE
LYOPHILIZATION OF CONJUGATES
LABEL AND COLOR CODING
PRE-COATED PLATE
ANTIGEN CONJUGATES
TRI-ELISA MOSQUITO COMPONENTS
COMMON CONJUGATE
COMMON SUBSTRATE
SINGLE ELISA COMPONENTS
MoAb PEROXIDASE CONJUGATES
SUBSTRATE
HUMAN IgG ASSAY COMPONENTS
ANTI-HUMAN IgG-PEROXIDASE
SUBSTRATE
PRE-COATED PLATE
DELIVERY OF PRE-COATED PLATES TO WRITZ
ANTIGEN CONJUGATES
ENZYME CONJUGATES
COMMON SUBSTRATE
WHOLE ASSAY
IN-HOUSE EVALUATION
SPOROZOITES IN MOSQUITOS
TRI-ELISA ASSAY
SINGLE ELISA ASSAY
HUMAN SERUM TESTING
TEST KITS TO WRITZ
FIELD TESTING BY WRITZ
SPOROZOITES IN MOSQUITOS
TRI-ELISA
SINGLE ELISA
HUMAN SERUM TESTING



BSI Corp.
9924 West Seventy Fourth Street
Eden Prairie, MN 55344
612-829-2700

Malaria Test Kit Introduction

This kit contains the components necessary for running three different malaria ELISAs. The Tri-enzyme ELISA and the Quantitative ELISA have been designed for the detection of *Plasmodium vivax*-210, *Plasmodium falciparum, and Plasmodium vivax* -247 type sporozoites isolated from the salivary glands of infected mosquitoes. The Human Serum ELISA has been developed to test human subjects for titers raised against the three types of *Plasmodium* sporozoites listed above.

Kit Abbreviations

HRP = Horseradish peroxidase enzyme.

TNT= 50 mM Tris, 150 m M NaCl, 0.05% Tween 20. Store up to one week at room temperature or one month at 4°C.

Malaria Test Kit Components (Store at 4°C)

- **2 Precoated Antibody Plates**
  Microtiter plates coated with 2 µg/ml each of *Plasmodium vivax*-210, *Plasmodium falciparum*, and *Plasmodium vivax*-247 monoclonal antibodies and stabilized with StabilCoat™ Immunoassay Stabilizer. Plates are supplied in foil packages containing desiccant.
- **8 Plate Sealers**
  Dynatech Laboratory plate sealers, Catalog # 001-010-3501.
- **1 lyophilized *P.vivax*-210 Antigen (red cap)**
  Plasmodium vivax -210 protein (NS1V20) conjugated to boiled casein.
  Contains 500 ng of lyophilized antigen. Reconstitute with 1 ml dH_2Obefore use.
- **1 lyophilized *P.falciparum* Antigen (yellow cap)**
  Plasmodium falciparum R32tet32 protein - boiled casein conjugate.
  Contains 500 ng of lyophilized antigen. Reconstitute with 1 ml dH_2O before use.
- **1 lyophilized *P.vivax* -247 Antigen (blue cap)**
  Plasmodium vivax-247 peptide $(Ala\text{-}9\text{-}Gly)_3$ conjugated to boiled casein.
  Contains 10 µg of lyophilized antigen. Reconstitute with 1 ml dH_2O before use.
- **1 lyophilized Enzyme Conjugate Cocktail**
  Contains a mixture of 160 µg *Plasmodium vivax* -210 antibody-alkaline phosphatase conjugate and 160 µg *Plasmodium falciparum* antibody-β- galactosidase conjugate. For use in Tri-enzyme ELISA. Reconstitute with 20 mls of diluted (1µg/ml) *P. vivax* 247 Enzyme Conjugate before use.
- **1 liquid*P.vivax* -210 Enzyme Conjugate**
  Plasmodium vivax antibody-horseradish peroxidase (HRP)conjugate. For use in the Quantitative ELISA. 20 ml supplied at use concentration (0.5 µg/ml) in SuperZym stabilizer +0.01% thimerisol.
- **1 liquid *P.falciparum* Enzyme Conjugate**
  Plasmodium falciparum antibody-HRP conjugate. For use in the Quantitative ELISA. 20 ml supplied at use concentration (0.5 µg/ml) in SuperZym stabilizer+0.01% thimerisol.
- **1 liquid *P.vivax* 247 Enzyme Conjugate**
  Plasmodium vivax-247 antibody-HRP conjugate. For use in theQuantitative ELISA.. 2 ml supplied at a 10x concentration (10 µg/ml) in SuperZym stabilizer+0.01% thimerisol.
- **1 liquid Anti-IgG Enzyme Conjugate**
  Anti-human IgG-Fc-horseradish peroxidase conjugate. For use in the Human Serum ELISA. 20 ml supplied at use concentration (0.5 µg/ml) in SuperZym stabilizer+0.01% thimerisol.
- **1 lyophilized Common Substrate**
  Reconstitute with 20ml of dH_2O before use in Tri-enzyme ELISA. Contains o-nitrophenyl galactopyranoside and phenolphthalein diphosphate in glycine buffer.
- **1 HRP Substrate**
  25 ml bottle of TMB, 3,3'5,5'-tetramethylbenzidine. Use as substrate for the Quantitative, Human Serum, or Tri-Enzyme ELISA.

Important tips for optimal assay performance

- **Use high quality deionized H_2O to reconstitute the lyophilized reagents**
- **For optimal assay sensitivity and reproducibility, we suggest performing all assay incubations at 37°C in an environmental shaker, with the exception of the HRP substrate incubations which should be performed at room temperature.** All assay incubations may be performed at room temperature, but the sensitivity and reproducibility may be reduced.
- **Do not expose the assay components to sunlight and do not perform the assays outdoors.**

The Tri-enzyme ELISA is a screening assay which enables the assayer to visually detect the presence of zero to three species of *Plasmodium* sporozoites in a single assay well by the development of zero to three different colors (red, yellow, and blue) where each color corresponds to a different *Plasmodium* sporozoite. (See figure 1).

The Tri-enzyme ELISA is a "sandwich" ELISA which works on the following principle: Precoated antibody plates provided in this kit are coated with a stable, dry mixture of the three different monoclonal capture antibodies developed against *Plasmodium vivax*-210, *Plasmodium falciparum, and Plasmodium vivax* -247 sporozoites. The antibody plates are reconstituted by soaking the plates with assay buffer for at least 15 minutes and then washing the plates with fresh buffer. Then mosquito triturate test samples, positive controls [circumsporozoite (CS) proteins or peptides conjugated to boiled casein] and negative controls (assay buffer or known negative mosquito triturate) are added to wells and incubated for one hour to allow the CS antigen, if present, to form an antibody-antigen complex with the appropriate antibody. Then the wells are washed and an enzyme conjugate cocktail is added which contains the *P. vivax*-210, *P. falciparum, and P. vivax*-247 monoclonal antibodies conjugated to three different enzymes (alkaline phosphatase, β-galactosidase and horseradish peroxidase, respectively). If one or more of the antigens is present in the well, the appropriate antibody-enzyme conjugate or conjugates will bind and complete the "sandwich." After another hour, the plate is washed and the substrate is added in two steps. The Step 1 Common Substrate Solution is a colorless solution which contains chromagenic substrates for alkaline phosphatase (AP) and β-galactosidase (β-gal). The Common Substrate Solution will produce a yellow color in the well if the *P. falciparum*-β-gal conjugate is present and a red color in the well if the *P. vivax*-210-AP conjugate is present. If both the *P. falciparum*-β-gal and *P. vivax*-210-AP conjugates are present, the well will be orange. After a 30 minute incubation with the Common Substrate Solution, a colorless Step 2 HRP Substrate Solution (TMB/H_2O_2) is added which produces a blue color in wells containing *P. vivax* -247-HRP conjugate. Simultaneously, the HRP Substrate Solution completely quenches all the red color and most of the yellow color (highly positive *P. falciparum* wells may retain some light yellow color). Therefore, if all three CS antigens are present, the well will be orange after the addition of the Step 1 Common Substrate Solution and blue (or possibly green if highly positive for *P. falciparum*) after addition of the Step 2 HRP Substrate Solution.

The Tri-enzyme ELISA is less sensitive than the Quantitative ELISAs that follow, and is intended to be used only as a screening tool. Once the type or types of sporozoites present in a sample have been identified, a Quantitative ELISA should be run for verification and quantitation of the sporozoite or sporozoites present.

THE TRI-ENZYME ELISA ASSAY

1. Add TNT buffer (100-200 µl/well) to Precoated Antibody Plates, soak at least 15-20 minutes. Meanwhile, prepare test samples and positive antigen controls Reconstitute positive antigen controls with 1 ml dH_2O and dilute as follows in TNT buffer.

P.vivax -210 Antigen:

Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
25 ng/ml = 50 µl stock plus 950 µl TNT buffer.

P. falciparum Antigen:

Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
25 ng/ml = 50 µl stock plus 950 µl TNT buffer.

P.vivax -247 Antigen:

Reconstituted stock =10 µg/ml. Dilute control to 500 ng/ml.
500 ng/ml = 50 µl stock plus 950 µl TNT buffer.

Mixture of all three antigens:

25 ng/ml *P.vivax* -210 Antigen + 25 ng/ml *P. falciparum* Antigen + 500 ng/ml *P.vivax*-247 Antigen: = 50 µl of each stock 850 µl TNT.

[Note- If only one plate is being run in the assay, the unused reconstituted antigen stocks may be stored refrigerated for up to 1 week. For longer term storage, store frozen].

2. Wash plates 3 times with 250 µl TNT buffer. Then add test samples (50-100 µl/well), negative controls (TNT buffer or negative mosquito triturate, 100 µl/well) and positive antigen controls (100 µl/well).

Incubate for 1 hour at 37°C.

3. Wash plates 3 times with 250 µl TNT buffer. Prepare the three enzyme conjugate mixture as given below and add the conjugate mixture to the plate (100 µl/well).

Enzyme Conjugate Mixture Preparation:

Dilute the vial containing 2 ml of 10 µg/ml *P.vivax*-247 Enzyme Conjugate with 18 ml of TNT+ a blocking protein (we recommend 1% BSA). Then use the diluted *P.vivax* -247 Enzyme Conjugate to reconstitute the lyophilized Enzyme Conjugate Cocktail. Mix well.

[Note- If only one plate is being run, reconstitute the Enzyme Conjugate Cocktail with 2 ml dH_2O. Then prepare the enzyme conjugate mixture by mixing 1 ml of reconstituted Enzyme Conjugate Cocktail, 1 ml of 10 µg/ml P.vivax -247 Enzyme Conjugate and 8 mls of TNT+blocking protein . Store the unused reconstituted Enzyme Conjugate Cocktail at 4° C and use within a few days for optimal activity].

Incubate for 1 hour at 37°C.

4. Wash plates 5 times with 250 µl TNT. Add the Common Substrate (100 µl/well).

Common Substrate Preparation:

Add 10 ml H_2O and mix vigorously; it may take 5-10 minutes for all of the lyophilized reagent to dissolve.

[Note- Store unused Common Substrate for up to 1 week at 4° C. Discard if the solution becomes cloudy or noticeably discolored (a light pink or yellow hue is acceptable)].

Incubate for 30 minutes at 37°C.

5. Identify wells containing *P. vivax* Antigen and *P. falciparum* Antigen.
P. vivax -210 Antigen = reddish or pink colored wells, absorbance may be read at 570 nm.
P. falciparum Antigen = yellow colored wells, absorbance may be read at 405 nm.
P. vivax -210 Antigen and *P. falciparum* Antigen mixture = orange, pinkish orange or yellowish orange colored wells; absorbance may be read at 405 and 570 nm.

6. Next, add the HRP Substrate. (Note -When the HRP Substrate is added, the red color will disappear and the yellow color will either lighten or disappear)

Incubate for 30 minutes at room temperature .

7. After 30 minutes, visualize wells as follows or read on a plate reader at 655nm.
Pvivax -247 Antigen = blue colored wells (or possibly green wells if the test sample contains a high level of *P. falciparum* antigen), absorbances may be read at 655 nm

Figure 1: Tri-ELISA

The tri-ELISA is to be used for screening mosquitoes in the presence of one, two, or three species of *Plasmodium*. It is a qualitative, noninstrumented sandwich ELISA, comprised of a polystyrene 96 well plate pre-coated with three capture antibodies (*P. vivax*-210, *P. falciparum* and *P. vivax*-247); malaria antigen peptides/proteins coupled to boiled casein (BC) [*P. vivax*-210-BC (○), *P. falciparum*-BC (■), and *P. vivax*-247-BC (▲)]; a common enzyme conjugate mixture; and a two step substrate. The assay steps are represented by the following diagram:

Precoated antibody plates are removed from their package, soaked with buffer for 15 minutes, and washed.

P.v-247 Ag+ *P.v*-210 Ag+ *P.f* Ag+

Next, BC-antigen controls and mosquito extract samples are added to separate wells and allowed to incubate 1 hour.

After a wash step, the common enzyme conjugate cocktail mixture is added and allowed to incubate for 1 hour.

γ_β =anti-*P.v*-247-peroxidase γ_β =anti-*P.v*-210-alkaline phosphatase γ_α =anti-*P.f*-βgalactosidase

Following a wash step, the common substrate solution is added. Wells containing *P.v*-210 antigen turn red, and wells containing *P.f* antigen turn yellow.

After 30 minutes, HRP substrate is added and incubated 30 minutes Wells containing *P.v*-247 antigen turn blue, while the red and yellow colors are quenched.

BLUE RED YELLOW

THE QUANTITATIVE ELISA

This kit contains the components for running three different quantitative ELISAs: the ***Plasmodium vivax* -210 sporozoite ELISA**, the ***Plasmodium falciparum sporozoite ELISA,*** *and the* ***Plasmodium vivax* -247 sporozoite ELISA** (see figure 2). Each of these assays is a sandwich assay similar to the tri-ELISA assay given above except that only one enzyme conjugate is added per ELISA [*P vivax*-210-HRP, *P. falciparum*-HRP, or *P.vivax*-247-HRP]. TMB/H_2O_2 is used as the substrate for the HRP conjugates, and the absorbances of the test samples and controls are read on a plate reader at 655 nm after 30 minutes. The amount of CS antigen in the test sample is quantitated by comparing the absorbance of the test sample to a standard curve of CS antigen-boiled casein (BC) conjugates. The number of sporozoites present per mosquito may then be estimated from the amount of CS antigen present. If the absorbance of the sample is higher than the highest absorbance on the standard curve, the sample should be diluted in assay buffer and run again.

QUANTITATIVE ELISA ASSAY

1. Add TNT buffer (100-200 µl/well) to Precoated Antibody Plates, soak at least 15-20 minutes. Meanwhile, prepare test samples and dilute the appropriate positive antigen control. Reconstitute positive antigen controls with 1 ml dH_2O and dilute in TNT buffer as follows.

   P.vivax 210 Antigen:

   Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
   25 ng/ml = 50 µl antigen plus 950 µl TNT buffer.
   Prepare a 200 pg/ml stock (20 µl of 25 ng/ml + 2.48 ml TNT)
   Next, serially dilute in two fold dilutions (1 ml sample +1 ml TNT) to 100, 50, 25, 12.5, 6.25, and 3.13 pg/ml.

   P.falciparum Antigen:

   Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
   25 ng/ml = 50 µl antigen plus 950 µl TNT buffer.
   Then prepare a 800 pg/ml stock (80 µl of 25 ng/ml + 2.42 ml TNT)
   Next, serially dilute in two fold dilutions (1 ml sample +1 ml TNT) to 400, 200, 100, 50, 25, and 12.5 pg/ml.

   P.vivax -247 Antigen:

   Reconstituted stock =10 µg/ml. Dilute control to 500 ng/ml.
   500 ng/ml = 100 µl antigen plus 1900 µl TNT buffer.
   Next, serially dilute to 250, 125, 62.5, 31.3, and 15.6 ng/ml

   [*Note- The unused reconstituted antigen stocks may be stored for 1 week at 4° C. For longer term storage, store frozen*].

   Incubate for 1 hour at 37°C.

2. Wash plates 3 times with 250 µl TNT buffer. Add test samples (50-100 µl/well), negative controls (TNT buffer or negative mosquito triturate, 100 µl/well) and positive antigen controls (100 µl/well). **Incubate for 1 hour at 37°C.**

3. Wash plates 3 times with 250 µl TNT buffer. Add the appropriate conjugate, 100 µl/well. The *P.vivax* -210 and *P.falciparum* Enzyme Conjugates are supplied at use concentration and may be directly added to the plate without dilution. The *P.vivax* -247 enzyme conjugate should be diluted in 18 mls TNT+a blocking protein (we recommend 1% BSA) prior to addition to the plate. **Incubate for 1 hour at 37°C**

4. Wash 5 times with 250 µl TNT. Add HRP substrate. **Incubate 30 minutes at RT** and read on a plate reader at 655 nm.

Figure 2: Quantitative ELISA

Components for the quantitative determination of each type of sporozoite in mosquitos are included in the kit. This assay was previously developed by Dr. Wirtz and associates; the only difference is that this assay will be performed using pre-coated antibody plates. The quantitative ELISA is also a sandwich assay but it does not differentiate between *Plasmodium* species in a single well.

Precoated antibody plates are removed from their package, soaked with buffer for 15 minutes, and washed.

P.v-247 Ag+ *P.v*-210 Ag+ *P.f* Ag+

Next, BC-antigen controls and mosquito extract samples are added to separate wells and allowed to incubate 1 hour.

After a wash step, the enzyme conjugate is added and allowed to incubate for 1 hour.

Y_δ =anti-*P.v*-247-peroxidase Y_β =anti-*P v*-210-peroxidase Y_α =anti-*P f*-peroxidase

Following a wash step, the HRP substrate solution is added and allowed to incubate 30 minutes. The plate is read using a microtiter plate reader.

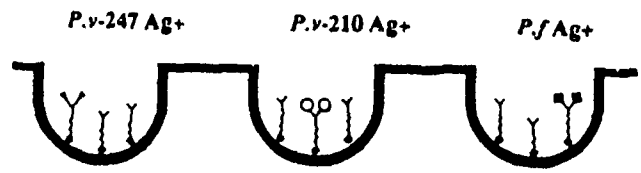
BLUE **BLUE** **BLUE**

Figure 3: Human Serum ELISA

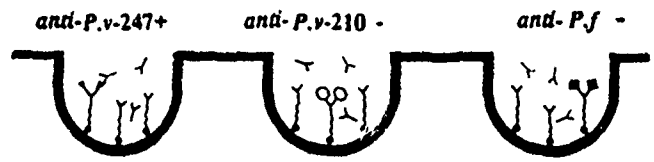
The Human Serum ELISA will be used to screen human serum samples for antibodies developed against circumsporozoite antigens. The pre-coated antibody plate will be used to capture the appropriate antigen, then the serum samples will be added followed by anti-human IgG enzyme conjugate and enzyme substrate.

Precoated antibody plates are removed from their package, soaked with buffer for 15 minutes, and washed.

Next, BC-antigen controls and mosquito extract samples are added to separate wells and allowed to incubate 1 hour.



Following a wash step, serum samples diluted 1:100 in buffer are added and incubated for 1 hour.

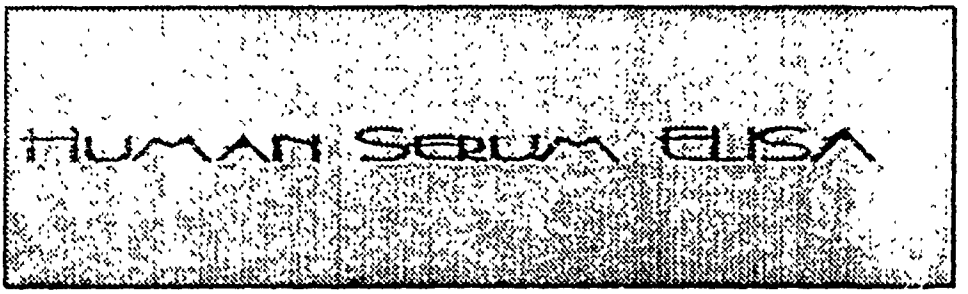


After a wash step, the enzyme conjugate is added and allowed to incubate for 1 hour.

Y = anti-human-IgG-peroxidase

Following a wash step, the HRP substrate solution is added and allowed to incubate 30 minutes. The plate is read using a microtiter plate reader.

BLUE NO COLOR NO COLOR



The **Human Serum ELISA** is used to test human serum samples for exposure to *Plasmodium vivax*-210, *Plasmodium falciparum*, or *Plasmodium vivax*-247 malaria. In this assay, precoated antibody plates are incubated with one of the CS antigen-BC conjugates. Then, human serum test samples and controls (positive and negative serum control samples) are added and incubated for 1 hour. If human antibodies against the CS antigen are present in the sample, the antibody will bind to the CS antigen-BC conjugate. The sample is then washed and a goat anti-human-IgG-Fc HRP conjugate is added which will bind to human antibodies present in the well. After incubation for one hour, HRP substrate is added and blue color will develop in wells containing positive serum samples. (See figure 3).

Human Serum ELISA

1. Add TNT buffer (100-200 µl/well) to Precoated Antibody Plates, soak at least 15-20 minutes. Meanwhile, dilute the appropriate positive antigen control. Reconstitute positive antigen controls with 1 ml dH_2O and dilute in TNT buffer as follows.

   P.vivax-210 Antigen:
   Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
   25 ng/ml = 50 µl antigen plus 950 µl TNT buffer.
   Then prepare a 200 pg/ml stock (200 µl of 25 ng/ml + 24.8 ml TNT)

   P. falciparum Antigen:
   Reconstituted stock = 500 ng/ml. Dilute control to 25 ng/ml.
   25 ng/ml = 50 µl antigen plus 950 µl TNT buffer.
   Then prepare a 800 pg/ml stock (800 µl of 25 ng/ml + 24.2 ml TNT).

   P.vivax 247 Antigen:
   Reconstituted stock =10 µg/ml. Dilute control to 500 ng/ml.
   500 ng/ml = 1000 µl antigen plus 19 ml TNT buffer.

   [*Note-Unused reconstituted antigen stocks may be stored for 1 week at 4° C. For longer term storage, store frozen*].

2. Wash plates 3 times with 250 µl TNT buffer. Then add positive antigen controls to both plate (100 µl/well).

   Incubate for 1 hour at 37°C .

3. Wash plates 3 times with 250 µl TNT buffer. Then add positive and negative serum controls as well as serum samples (100 µl/well).

   Incubate for 1 hour at 37°C.

4. Wash plates three times with 250 µl TNT buffer. Add the Anti-IgG Enzyme Conjugate to the plates,100 µl/well.

   Incubate for 1 hour at 37°C.

5. Wash plates five times with 250 µl TNT. **Add HRP Substrate and incubate for 30 minutes at room temperature.** Read plates after 30 minutes at 655 nm.